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The role of glutamine metabolism for host defense against *Mycobacterium tuberculosis* infection

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Abstract

Background Rewiring cellular metabolism is important for activation of immune cells during host defense against *Mycobacterium tuberculosis*. Glutamine has been implicated as an immunomodulatory nutrient, but its role in response to *M. tuberculosis* is unknown.

Methods We assessed expression of glutamine pathway genes in *M. tuberculosis* infected macrophages and blood transcriptomics from individuals with latent or active tuberculosis. Subsequently, we studied the effect of blocking glutaminolysis on *M. tuberculosis*-induced cytokines. Finally, we examined whether polymorphisms in genes involved in the glutamine pathway influence *M. tuberculosis*-induced cytokines in a cohort of 500 individuals.

Results Glutamine pathway genes were differentially expressed in infected macrophages and patients with active tuberculosis. Human peripheral blood mononuclear cells stimulated with *M. tuberculosis* displayed decreased cytokine responses (IL-1 β , IFN- γ , and IL-17) when medium was devoid of glutamine. Specific inhibitors of the glutamine pathway led to decreased cytokine responses, especially T-cell cytokines (IFN- γ , IL-17, and IL-22). Finally, genetic polymorphisms in glutamine metabolism genes (including *GLS2*, *SLC1A5*, and *SLC7A5*) influenced ex-vivo cytokine responses to *M. tuberculosis*, especially for T-cell cytokines.

Conclusions Cellular glutamine metabolism is implicated in effective host responses against *M. tuberculosis*. Targeting immunometabolism may represent new strategies for TB prevention and/or treatment.

Keywords: tuberculosis; *Mycobacterium tuberculosis*; immune response; cytokines; immunometabolism; glutamine metabolism.

Introduction

Changes in cellular metabolism play a vital role in the initiation and regulation of the immune response. Once infected, immune cells undergo rewiring of cellular metabolism to adapt to the infection and generate sufficient energy to carry out host defense functions. Metabolic reprogramming is also important for host responses against *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB). *M. tuberculosis* induces a switch in cellular metabolism in peripheral blood mononuclear cells (PBMCs) towards aerobic glycolysis. This metabolic switch is mediated through the AKT-mTOR pathway, and inhibition of glycolysis results in reduced cytokine production and mycobacterial killing (1, 2). We have recently identified other metabolic pathways, including glutathione and pyrimidine metabolism, to be differentially expressed at tissue level in tuberculosis patients, and to alter Th1 and Th17 production in response to *M. tuberculosis* (3). However, the role of other metabolic pathways on the immune response against *M. tuberculosis* remains largely unknown.

Glucose can be used to generate ATP either through glycolysis, which involves the conversion of glucose to pyruvate and thereafter through fermentation to lactate in the cytoplasm, or by oxidation of pyruvate in the tricarboxylic acid (TCA) cycle, which generates nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) to fuel oxidative phosphorylation (OXPHOS) (4). Glutamine, a non-essential amino acid, can be used as an additional carbon source to fuel the TCA cycle by a process called glutaminolysis. To accomplish this, glutamine is converted to glutamate by the enzyme glutaminase, and the enzyme glutamate dehydrogenase (GDH) mediates the conversion from glutamate to α -ketoglutarate (α -KG), which is a substrate for the TCA cycle. The importance of glutamine metabolism during activation of host defense has been described in other infections. For instance, during cytomegalovirus infection, glutamine consumption increases, and infected cells become dependent upon glutamine for ATP production (5). In addition, glutaminolysis is essential for the induction of trained immunity (innate immune memory) in monocytes (6).

The aim of this study was to examine the relevance of glutamine metabolism in host responses against *M. tuberculosis*. To this purpose, we assessed whether genes involved in glutaminolysis are modulated during *M. tuberculosis* infection; we studied the effect of blocking glutaminolysis on *M. tuberculosis*-induced cytokines; and we examined if variation in glutamine pathway genes acted as quantitative trait loci (QTLs) for ex-vivo *M. tuberculosis*-induced cytokines in a cohort of 500 individuals.

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Methods

Peripheral blood mononuclear cell (PBMC) isolation and stimulation

Informed consent from healthy volunteers was obtained for use of their blood for scientific purposes, as approved by the Ethics Committee of Radboud University Medical Centre, Nijmegen, the Netherlands. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers (Sanquin Bloodbank, Nijmegen, the Netherlands). Isolation was performed using Ficoll-Paque, involving separation by a density gradient followed by three wash steps in cold PBS and resuspension in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10 µg/mL gentamicin, 2 mM GlutaMAX (except for the glutamine depletion experiment), and 5.5 mmol/L glucose. After isolation, PBMCs (5×10^5 cells/well) were seeded in 96-well round-bottom plates and stimulated with *M. tuberculosis* H37Rv lysate (1 µg/mL), *Candida albicans* (1×10^6 copies/mL), *Staphylococcus aureus* (1×10^6 copies/mL), PHA (10 µg/mL) or culture medium. The cultures were incubated for 24 h or 7 days at 37°C in 5% CO₂, and supplemented with 10% human pooled serum in case of a 7-day stimulation, after which supernatants were collected and stored at -20°C. In some experiments, cells were pre-incubated (before stimulation) for 1 h with 600 µM gamma-L-Glutamyl-p-Nitroanilide (GPNA), 10 µM 6-Diazo-5-oxo-L-norleucine (DON), 30 µM C968, 50 µM BPTES (Sigma), or 5 mM aminooxyacetate (AOA).

Cytokine and lactate measurements

Enzyme-linked immunosorbent assays (ELISAs) were performed on supernatants from the PBMC stimulations following the manufacturer's protocols for measuring cytokines in supernatants. The concentrations of interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) (R&D Systems, Minneapolis, Minnesota, USA), IL-10 and IL-6 (Sanquin, Amsterdam, the Netherlands) were measured after 1 day incubation and the concentrations of IFN-γ (Sanquin, Amsterdam, the Netherlands), IL-17 and IL-22

(R&D Systems, Minneapolis, Minnesota, USA) were measured after 7 days. Lactate concentrations were measured after 1 day of incubation using Fluorometric Quantification Assay Kits (Biovision).

Metabolite measurements

After cell culture, cells were lysed in 0.5% Triton-X in PBS. Concentrations of fumarate, glutamate, pyruvate, α -KG, and malate were determined by commercial kits (Sigma), according to the instructions of the manufacturer.

Cell death assay

To measure the effect of the tested inhibitors on cell death, annexin V-FITC conjugate (Av, BioVision, Milpitas, California, USA) and propidium iodide (PI) were added after stimulation. Annexin V stains phosphatidylserine translocating from the inner to the outer leaflet of the membrane, marking early apoptosis. PI stains nuclei from cells that are permeable, reflecting cell death, either from advanced apoptosis or necrosis. Flow cytometric analysis using Cytomics FC500 was performed to distinguish Av- /PI- (alive), Av+ /PI- (early apoptotic) and Av+ /PI+ (advanced apoptotic / necrotic) populations.

In-vitro gene expression dataset

CD14⁺ monocytes were isolated from PBMCs by magnetic cell sorting with anti-CD14-coated MACS microbeads (Miltenyi Biotec) and seeded in tissue culture-treated flasks (Corning). After 6 days of differentiation in the presence of 50 ng/mL M-CSF, macrophages were harvested using trypsin and transferred to tissue culture-treated 24-well plates (Corning) with 300,000 macrophages per well. Macrophages were incubated O/N at 37°C in 5% CO₂ and subsequently infected with the H37Rv strain of *M. tuberculosis*. Bacterial density was determined by measuring optical density at 600 nm (OD₆₀₀) and the bacterial suspension was diluted to a concentration of 30 x 10⁶ bacteria/mL to infect the macrophages at a multiplicity of infection (MOI) of 10. 100 μ L of the bacterial suspension was added to the cell cultures, after which the plates were centrifuged for 3 minutes at 800 rpm and

incubated at 37°C in 5% CO₂. After 60 minutes the plates were washed with culture medium containing 30 µg/mL gentamicin to kill extracellular bacteria and subsequently incubated O/N at 37°C in 5% CO₂ in medium containing 5 µg/mL gentamicin. Cells were lysed by direct addition of TRIzol to the wells, followed by RNA extraction. RNAseq libraries were prepared from 1 µg RNA using the TruSeq RNA sample preparation kit v2 (Illumina) according to the manufacturer's instructions, and these libraries were subsequently sequenced on a HiSeq 2000 sequencer (Illumina) using single-end sequencing of 50 bp, upon pooling of 10 samples per lane.

In-vivo gene expression dataset

We downloaded previously published whole blood microarray data from patients with pulmonary TB (culture positive), latent TB and healthy controls (TST and IGRA negative) (7). Each microarray was scaled to the median average intensity for all samples (per-chip normalization). These data were obtained from Gene Expression Omnibus (GEO) under accession number GSE19491.

Cytokine quantitative trait loci (cQTL) analyses

Single nucleotide polymorphisms (SNPs) in sixteen genes from the glutamine pathway were identified. SNPs were then mapped ($p < 0.05$ cut-off) for cytokine quantitative trait loci (QTLs) in the 500FG cohort. This cohort consists of 500 healthy individuals of Dutch European ancestry from the Human Functional Genomics Project (www.humanfunctionalgenomics.org), from which peripheral blood mononuclear cells (PBMC) or macrophages have been stimulated by heat-killed *M. tuberculosis* and the secretion of IFN- γ , IL-17, IL-22 (7 days post-stimulation) and IL-1 β , IL-6 and TNF- α (24h post-stimulation) measured, as previously described (8). To identify cQTLs, raw cytokine levels were first log transformed then mapped to genotype data using a linear regression model with age and gender as covariates. P values were obtained using linear regression analysis of cytokine on genotype data, as previously described (8). These cQTL data are publically available at www.humanfunctionalgenomics.org.

Statistical analysis

The non-parametric Wilcoxon matched-pairs signed rank test was used to compare cytokine and metabolite data from the in-vitro experiments between groups. P-values of 0.05 or less were considered statistically significant. These statistical analyses were performed using GraphPad Prism 5.03. To analyze the gene expression datasets, principal component analyses (PCA) were performed on centered, scaled data using 'prcomp'. The heatmap was generated using 'heatmap', and samples were ordered using hierarchical clustering. All analyses performed on gene expression data were performed using R 3.2.2 (<http://www.R-project.org>).

Results

Transcriptional changes in genes involved in glutamine metabolism *in vivo* and in *M. tuberculosis*-infected macrophages

We examined whether *M. tuberculosis* infection leads to an altered expression of genes of the glutamate pathway, using in-vitro infected macrophages and available blood transcriptome data from patients and controls. Based on expression of genes from the glutamine pathway, *M. tuberculosis*-infected macrophages could be clearly separated from uninfected macrophages using principal component analysis (**Figure 1a**). To further understand the class separation between infected versus uninfected macrophages, samples were clustered using unsupervised hierarchical clustering method using the gene expression of these glutamine-pathway genes. Two major clusters were found, one containing the *M. tuberculosis*-infected macrophages, and one with the uninfected macrophages (**Figure 1b**). Overall, *M. tuberculosis* infection led to higher expression of genes involved in transport and glutamine utilization. In addition, *M. tuberculosis* infection decreased expression of several genes involved in the TCA cycle, whereas ME2, which converts malate into pyruvate, and GLUD2, which converts glutamine to α -ketoglutarate, were specifically upregulated. Pulmonary TB patients and uninfected control subjects were clearly separated based on their whole blood expression of these glutamine-pathway genes, while individuals with latent tuberculosis infection (LTBI) were spread throughout the plot (**Figure 1c**).

M. tuberculosis-induced cytokines are decreased after glutamine depletion

To study the effect of *M. tuberculosis* on glutamine metabolism, we assessed whether *M. tuberculosis* stimulation increased the production of glutamine-derived metabolites *in vitro*. Peripheral blood mononuclear cells (PBMCs) stimulated with *M. tuberculosis* showed a significant increase in intracellular glutamate and malate (Wilcoxon signed-ranked test, $p = 0.019$ and 0.022 , respectively), a strong increase in α -KG, and no difference in fumarate and pyruvate (**Figure 2a**).

Subsequently, we examined the effect of glutamine deprivation on the cytokine production in response to *M. tuberculosis*. Glutamine depletion resulted in a significant decrease in IL-1 β , IFN- γ , and IL-17 produced by PBMCs (Wilcoxon signed-ranked test, $p = 0.004$, 0.020 , and 0.037 , respectively) (**Figure 2b**). To study whether this decrease in cytokines upon glutamine deprivation was linked to lactate production, lactate levels were measured in supernatants from cultured monocytes and PBMCs. No differences in lactate were observed (**Figure 2c**), suggesting that glutamate is not a fuel for lactate production upon stimulation with *M. tuberculosis*.

Inhibition of glutamine transport and metabolizing enzymes decrease cytokine production

To determine which steps of glutamine utilization influence cytokine production by *M. tuberculosis*-stimulated PBMCs, pharmacologic inhibitors were added during culture. A schematic diagram indicating the site of action of the different inhibitors is depicted in **Figure 3**. Firstly, addition of GPNA, a competitive inhibitor of the glutamine uptake via SLC1A5 glutamine transporter, led to a decrease in the production of TNF- α and IL-17. Next, the effects of inhibition of glutaminase, an enzyme responsible for the conversion of glutamine into glutamate, the first step of glutaminolysis, was examined using three different compounds: DON, BPTES and C968. Addition of DON, a broad spectrum glutaminase inhibitor, significantly lowered the production of IL-17, IFN- γ , and IL-22 during stimulation of PBMCs, but did not affect any of the monocyte-derived cytokines. The effects of DON are not specific for *M. tuberculosis*-induced cytokines, as *C. albicans*-induced IL-17 and IL-22 are also affected by glutaminase inhibition (**Supplementary Figure 1**). BPTES and C968 are both specific inhibitors of mitochondrial glutaminase (GLS1). BPTES inhibited the production of IL-10, IL-17 and IL-22, while C986 suppressed the release of IL-1 β , IL-10, IL-17, IFN- γ , and IL-22. Finally, we examined the conversion of glutamate into α -ketoglutarate, using AOA, which inhibits aminotransferase. Almost all cytokines tested were suppressed by AOA, except for IL-10. The effects of these inhibitors on the cytokine production are summarized in **Figure 4**.

The impact of genetic variants on glutamine-dependent cytokine responses

Lastly, we examined whether genetic variation in genes involved in the glutamine pathway affects host response to *M. tuberculosis*. To do so, we tested if common genetic variants (minor allele frequency $\geq 5\%$) in glutamine-related genes are associated with differences in cytokine secretion in response to *M. tuberculosis* stimulation, using a cohort of 500 healthy individuals from the Human Functional Genomics Project (www.humanfunctionalgenomics.org). We identified 13 cytokine quantitative trait loci (cQTLs) in 7 genes related to glutamine metabolism (**Table 1**). The strongest cQTLs were identified in genes encoding for the high affinity glutamine receptor, SLC1A5, and two enzymes involved in the conversion of glutamine to glutamate, GLS and GLUD. Finally, we investigated the known effects of the identified QTLs on gene expression. This was done using the publically available cohort of Westra *et al.* who performed an expression QTLs (eQTLs) meta-analysis in non-transformed peripheral blood samples (9). Four of our identified cytokine QTLs were also known eQTLs (**Table 1**). This means that these genetic variants regulate both gene expression and cytokine production, suggesting that these genes might mediate the regulation of cytokines.

Discussion

A growing body of evidence supports the importance of cellular metabolism during immune activation and host defense against tuberculosis. For the first time, we examined the role of glutamine metabolism in *M. tuberculosis* infection. Transcriptional changes in glutamine-related genes were evident *in vitro* in *M. tuberculosis* infected macrophages and cells from pulmonary tuberculosis patients compared to healthy subjects. Glutamine depletion or glutamine pathway inhibitors predominantly reduced T-helper cell cytokine production. These findings were substantiated by the identification of SNPs in genes of the glutamine pathway that affected cytokine production in response to *M. tuberculosis* stimulation of human PBMCs. Together, these data underline the importance of glutamine metabolism in tuberculosis.

Interestingly, most glutamine metabolism inhibitors appeared to affect stronger the T cell-derived cytokine production (IFN- γ , IL-17, and IL-22) compared to monocyte-derived cytokines (TNF- α , IL-6, IL-1 β , and IL-10), suggesting a more prominent role for glutamine metabolism in the differentiation of T helper cells. This is supported by data from Nakaya *et al*, who showed that stimulation of naïve CD4⁺ T cells triggered glutamine uptake, and that glutamine was required for the differentiation of naïve T cells to Th1 and Th17 inflammatory T cells (10). The critical role for T cell immunity in the control of *M. tuberculosis* is evident, as defects in Th1 cytokine production, particularly IFN- γ , are risk factors for infection and disease progression (11). A balance between Th1 and Th17 responses is essential to control bacterial growth and limit immunopathology, as a shift of the response towards excessive IL-17 production may cause extensive neutrophil recruitment and tissue damage (12).

Genes involved in the glutamine pathway were differentially expressed after *M. tuberculosis* stimulation *in vitro*. We also observed genetic polymorphisms in glutamine pathway genes, including GLS2, SLC1A5 and SLC7A5, that controlled *M. tuberculosis*-induced cytokine production, which further strengthens the role of glutamine metabolism in the host defense against *M. tuberculosis*.

This is not the first study that observed immunomodulatory effects of glutamine. In an experimental cerebral malaria study, inhibition of glutamine metabolism improved survival of mice by inhibiting CD8⁺ T cell effector functions (13). In addition, glutaminolysis is essential for the induction of innate immune memory, termed trained immunity (6). In a murine study, a sustained trained immunity response was induced by β -glucan through modulation of hematopoietic stem and progenitor cells (HSPCs), which was associated with adaptations in glucose metabolism and cholesterol biosynthesis (Mitroulis cell 2018). Interestingly, intravenous BCG vaccination also led to changes in HSPCs, and these epigenetically modified macrophages provide better protection against *M. tuberculosis* infection (Kaufmann 2018 cell). Therefore, targeting the glutamine pathway might be a promising approach to modulate the immune response against *M. tuberculosis* through modulation of trained immunity.

Our study has some limitations. The cytokines studied in our in-vitro assays and the cQTL analysis represent only one component of the host immune response against *M. tuberculosis* infection. The role of glutamine metabolism on other aspects of the immune response, for example mycobacterial killing or phagocytosis, have not been explored in this study. Also, the relevance of glutamine metabolism during in-vivo infection is yet to be fully explored. Future studies should investigate the role of glutamine metabolism in live infection models, and study its role in susceptibility to tuberculosis in infected patients. In addition, tracer infusion studies could help unravel the in-vivo aspects of glutamine metabolism upon infection (14).

In conclusion, our study has identified glutamine metabolism as a fundamental metabolic process needed to mount an effective host response against *M. tuberculosis*. Earlier, we have identified other metabolic pathways, including glutathione and pyrimidine metabolism, to be relevant for host responses in tuberculosis (3). These studies and others underline the relevance of cellular metabolism for the response of immune cells to *M. tuberculosis*, and its potential role in developing new preventive or therapeutic strategies for tuberculosis.

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Conflict of interest

We declare that we have no conflicts of interest.

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Figure legends

Figure 1. *M. tuberculosis* induces transcriptional changes in genes involved in glutamine metabolism

(A) Principal component analysis of the glutamine gene signatures of macrophages stimulated with either live or dead *M. tuberculosis*, with proportion of variance per principal component indicated between brackets.

(B) Heatmap of gene expression pattern of glutamine genes in in vitro-stimulated macrophages. Samples (unstimulated or infected) were ordered by hierarchical clustering.

(C) Principal component analysis of the glutamine whole blood gene signatures of microarray data from a publically available cohort from South Africa, including pulmonary TB patients (PTB), latently infected individuals (LTBI), and healthy controls.

Figure 2. *M. tuberculosis*-induced cytokines are decreased after glutamine depletion

(A) PBMCs were stimulated with *M. tuberculosis* for 24h and intracellular glutamine metabolites were measured afterwards.

(B) PBMCs were stimulated with *M. tuberculosis* in the presence (black bars) or absence (grey bars) of glutamine and extracellular cytokines were measured after 24h of stimulation (TNF- α , IL-1 β , IL-6 and IL-10) or 7 days (IFN- γ , IL-17 and IL-22).

(C) PBMCs were stimulated with *M. tuberculosis* in the presence (black bars) or absence (grey bars) of glutamine and extracellular lactate was measured after 24 h.

Figure 3. Schematic diagram of metabolic pathways and site of action of inhibitors

The schematic is adapted from Hensley *et al.* (15). Metabolites are depicted in black, enzymes or receptors in blue, and inhibitors used in the in-vitro experiments in red.

Figure 4. Inhibition of glutamine transport and metabolizing enzymes decrease *M. tuberculosis*-induced cytokine production

PBMCs were stimulated with *M. tuberculosis* in the absence (grey bars) of presence (black bars) of specific inhibitors (GPNA, DON, BPTES, C968 or AOA) and extracellular cytokines were measured after 24h (TNF- α , IL-1 β , IL-6 and IL-10) or 7 days (IFN- γ , IL-17 and IL-22) of stimulation.

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Table 1: cytokine QTL results

Gene	SNP	T-cell cytokines			Monokines		eQTL	Gene function
		IFN- γ	IL-17	IL-22	IL-1 β	IL-6		
GLS2	rs2657879			0.002			2,22E-05	Converts Gln to Glu
GCLM	rs3112831		0.034				-	Converts Glu & Cys to Glutathione
GLUD1	rs760113	0.038					-	Converts Glu to aKG
	rs9864	0.027					-	
	rs77879311		0.005				-	
GPT	rs1063739					0.020	3,07E-51	Converts Ala & 2-oxoglutarate to pyruvate & Glu
	rs4251691					0.023	-	
SLC1A5	rs3027961					0.006	1,04E-10	High affinity L-glutamine transporter, takes up glutamine
SLC3A2	rs12808829	0.036					-	Antiporter. Exchanges intracellular Gln for extracellular Leu
	rs6856		0.046		0.021		-	
	rs4963245					0.036	-	
SLC7A5	rs34779002			0.024			-	Antiporter. Exchanges intracellular Gln for extracellular Leu
	rs2303772			0.022			2,34E-07	

Figure 1.

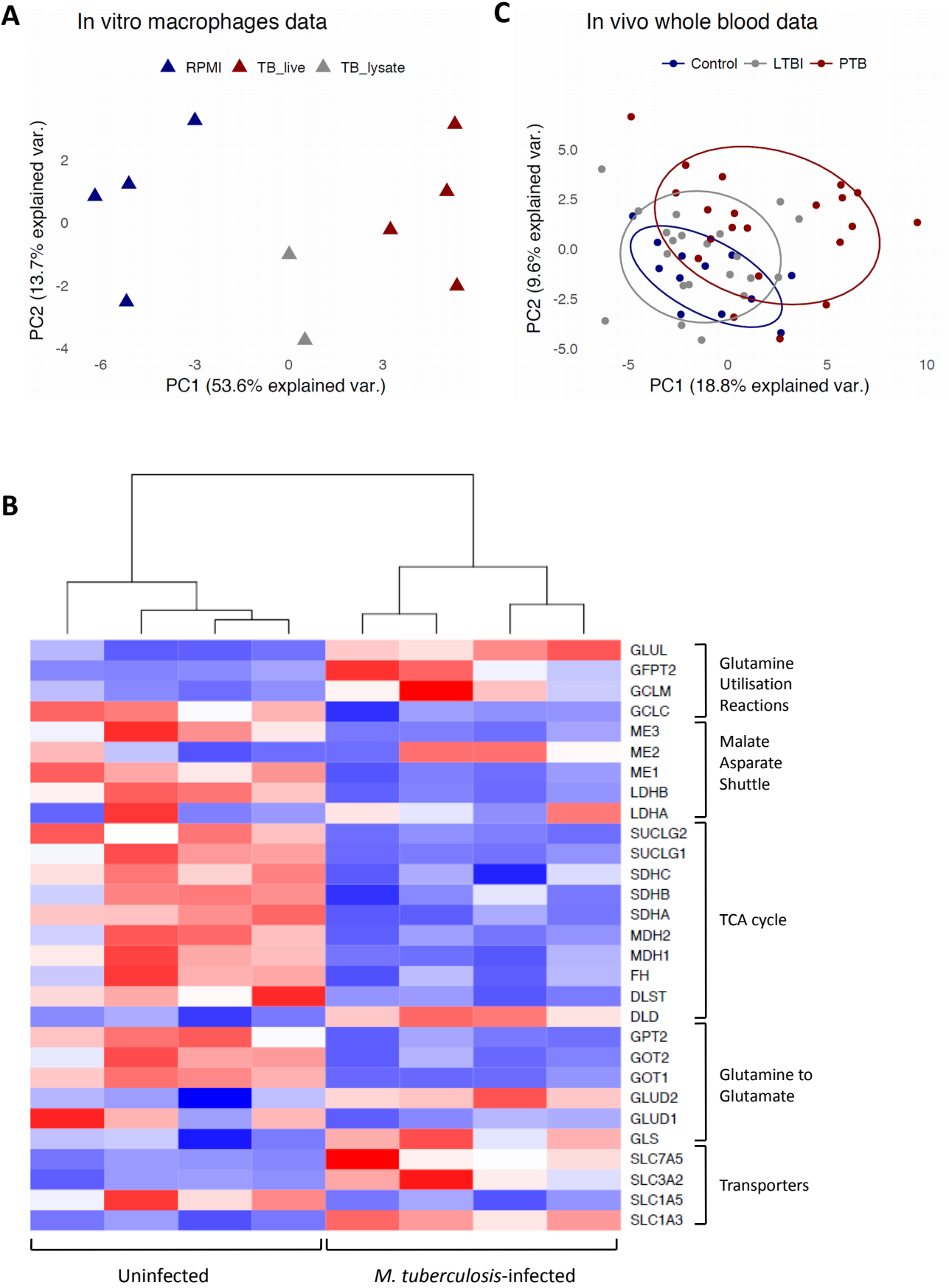
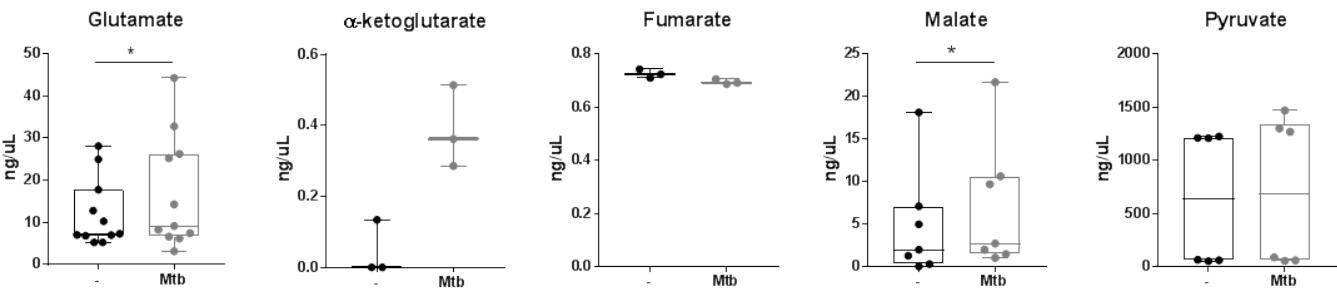
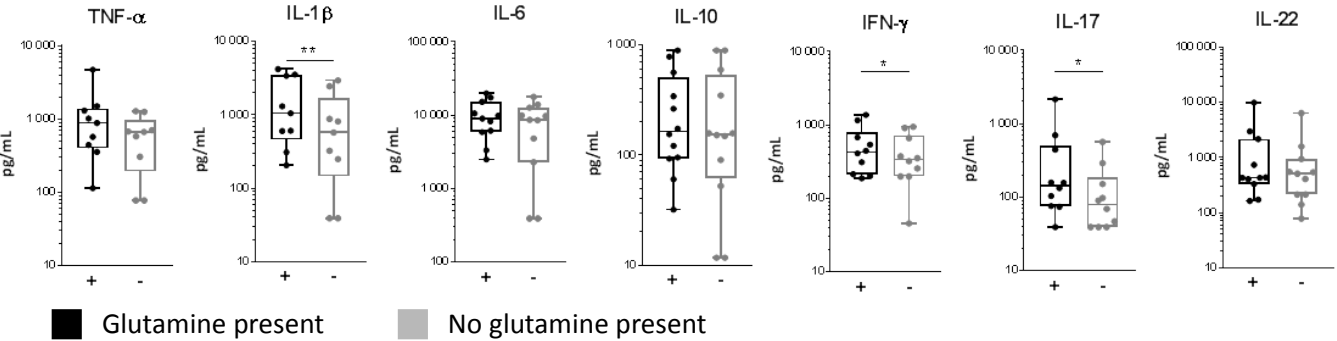


Figure 2.

A



B



C

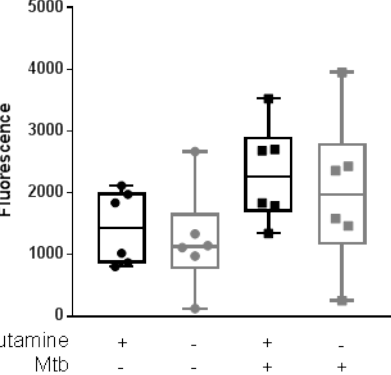


Figure 3.

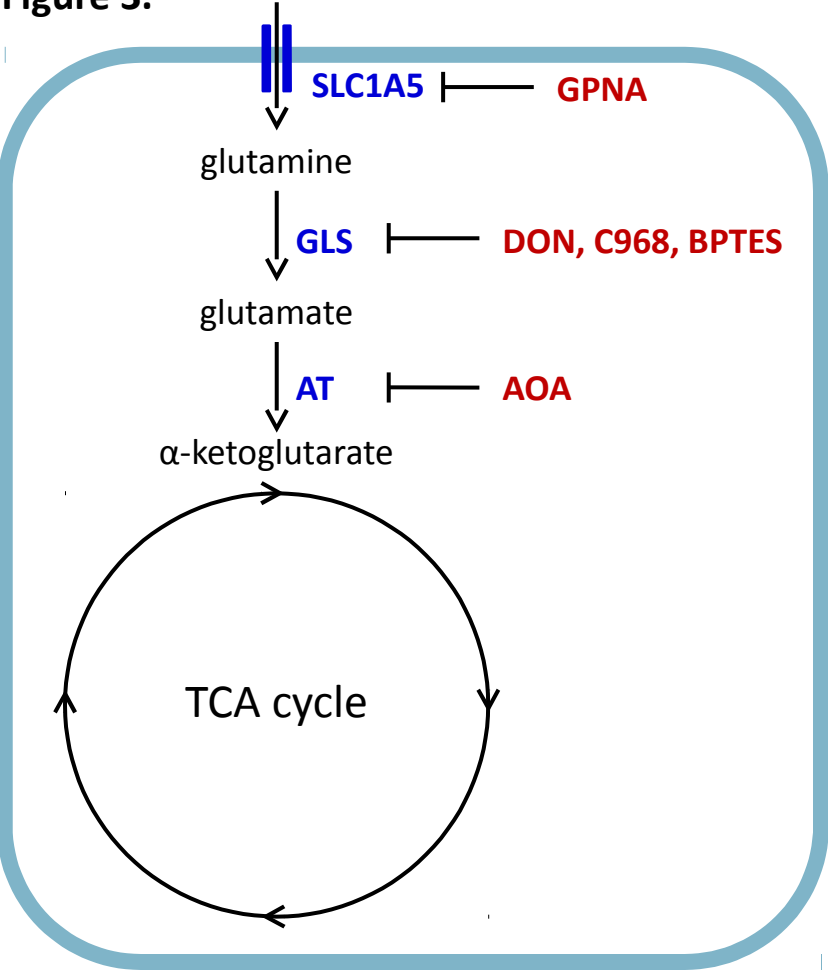


Figure 4.

